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(54) Title: METHODS FOR TREATING HUMAN CANCERS

(57) Abstract

The present invention provides methods for determining the appropriate therapy and/or prognosis for a cancer patient. The invention also provides methods for identifying compounds which will aid in the treatment of cancer.

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METHODS FOR TREATING HUMAN CANCERS

Background of the Invention

This invention relates to the treatment and prognostic assessment of neoplasms, in particular, cancer.

Cancer accounts for one fifth of the total mortality in the United States, and is the second leading cause of death after cardiovascular diseases such as myocardial infarction and stroke. A majority of human cancers exhibit diminished expression of the functional *p53* gene product (Jacks and Weinberg, Nature 381: 643-644, 1996). The pleiotropic disorder, Li-Fraumeni syndrome, which is characterized by a marked increase in breast cancer, sarcomas of soft-tissue and bone, brain tumors and other neoplasms, is caused by a mutation in the *p53* gene. Mice deficient in P53 are similarly prone to cancers, which are mainly thymic lymphomas (Donehower *et al.*, Nature 356: 215-221, 1992; Jacks *et al.*, Curr. Biol 4: 1-7, 1994).

It has been suggested that the ATM and P53 protein interact in acute radiation toxicity (see, for example, Meyn *et al.*, Canc. Res. 55: 5991-6001, 1995; Enoch *et al.*, Trends Biochem. Sci. 20: 426-430, 1995).

Summary of the Invention

In general, the invention features improved methods for the treatment and prognosis assessment of P53-related cancers.

In a first aspect, the invention provides a method for treating a neoplasm characterized by decreased levels of functional P53 protein. The method involves inducing a decreased level of biological activity of an ATM protein in cells of the neoplasm to enhance the sensitivity of the neoplasm to irradiation,

and exposing the neoplasm to a neoplastic cell-killing amount of irradiation. In one embodiment, the neoplasm is derived from a bone marrow cell, a gastrointestinal cell, or a fibroblast.

In another embodiment of the first aspect of the invention, the induction of a decreased level of biological activity of the ATM protein is carried out by introduction into the neoplasm of a neutralizing antibody specific for an ATM protein family member. In another embodiment, the induction is carried out by introduction into the neoplasm of ATM family member polypeptide fragments or mutants capable of competitively inhibiting the biological activity of the ATM family member protein. The induction may also be carried out by introduction into the neoplasm of nucleic acid capable of expressing ATM polypeptide fragments or mutants capable of competitively inhibiting the biological activity of the ATM protein. The induction may also be carried out by introduction into the neoplasm of antisense ATM nucleic acid which hybridizes to the ATM gene or mRNA, thereby inhibiting expression of the ATM protein.

In a second aspect, the invention provides a method for screening a compound for potential use as a anti-cancer therapeutic agent useful to treat cancers characterized by decreased levels of functional p53 protein. The method includes contacting the compound with an ATM protein, and then determining whether the compound reduces a biological activity of the ATM protein. The reduction of ATM protein biological activity by the compound indicates potential efficacy of the compound as an anti-cancer agent.

In a third aspect, the invention provides a method for determining whether a compound is capable of reducing the biological activity of an ATM protein. The method includes administering the compound and anti-cancer therapy to a provided mammal having a neoplasm characterized by reduced

p53 expression and then determining whether the therapeutic response of the mammal to the anti-cancer therapy is enhanced by the compound compared to the response of the mammal to the therapy in the absence of the compound, where an enhanced response indicates that the compound decreases the biological activity of the ATM protein.

In one embodiment of the third aspect of the invention, the mammal is a rodent. In another embodiment, the mammal may be immunocompromised. In yet another embodiment to the third aspect of the invention, the anti-cancer therapy is administration of γ-irradiation or a chemotherapeutic anti-cancer agent.

In a fourth aspect, the invention provides a method for determining whether a neoplasm will respond favorably to irradiation, which involves measuring the level of expression or biological activity of ATM protein in cells of the neoplasm, where a level lower than the level in cells of a fibrosarcoma or other cancer indicates a probable favorable response to irradiation.

By "ATM protein" is meant any member of the family of protein kinases whose members have an amino acid sequence identity of 25% to 60% to the p110 lipid kinase subunit of Phosphatidyl Inositol (PI) 3 kinase, as well as protein kinase activity. The four known members of this family are ATM, ATR (also known as FRP), FRAP (also known as mTOR, RAPT, and RAFT), and DNA-PK.

By "immunocompromised" is meant an animal incapable of mounting a normal immune response. Such an animal may be treated with an immunosuppressive drug (*e.g.*, rapamycin), may have a genetic defect (*e.g.*, a-thymic nude mice), or may be infected with a pathogen that suppresses the immune system (*e.g.*, HIV).

By "neutralizing antibody" is meant an antibody that interferes with any of the biological activities of an ATM family member polypeptide, particularly the protein kinase activity of an ATM family member. The neutralizing antibodies specific toward an ATM family member preferably reduce the protein kinase activity of a ATM family member polypeptide by at least 50%, more preferably by at least 70%, and most preferably by 90% or more. Any standard assay of protein kinase activity may be used to assess potentially neutralizing antibodies.

By "protein" or "polypeptide" is meant any chain of more than two amino acids, regardless of post-translational modification such as glycosylation or phosphorylation.

By "antisense sequence" is meant a nucleic acid sequence that is sufficiently complementary to a region of the coding strand of a gene to allow hybridization and concomitant inactivation of the gene *in vivo*.

Brief Description of the Drawings

Fig. 1A is a graph showing the percentage of increased apoptosis in thymocytes from *p53*-/-(hatched triangles), *atm* -/- (hatched squares), *atm*-/- ; *p53*-/- (crosses), and wild-type (closed diamonds) mice following treatment of the thymocytes with 5 Gy irradiation. The double null thymocytes (crosses) were as resistant to gamma irradiation-mediated apoptosis as p53 null thymocytes (hatched triangles). Error bars are indicated.

Fig. 1B is a graph showing the number of viable thymocytes from *p53*-/- (hatched triangles), *atm*-/-(hatched squares), *atm*-/-; *p53*-/- (crosses), and wild-type (closed diamonds) mice following treatment of the thymocytes with dexamethasone. Error bars are indicated.

Fig. 2A is a Western blotting analysis of whole cell protein extracts from *atm* null and wild-type mice at different time points following 5 Gy irradiation probed with P53-specific antibodies.

Fig. 2B is a schematic diagram of a model indicating that irradiation-induced thymocyte apoptosis is mediated through at least two P53-dependent pathways, only one of which involves ATM.

Fig. 3A is a graph showing a mortality curve of *atm*-/- (closed diamonds), *atm*+/-; *p53*-/- (open diamonds), *atm*-/-; *p53*+/- (darkly outlined open squares), and *atm*-/-; *p53*-/- (lightly outlined open squares) mice for 100 days.

Fig. 3B is a Southern blotting analysis of tail and tumor (tum) DNA from *atm*-/-; *p53*+/- mice, 1262, 1292, and 1249, probed with radiolabelled nucleic acid corresponding to the *p53* gene.

Fig. 4A is a graph showing a mortality curve of 4 week old wild-type (lightly outlined open squares), *p53*-/- (closed squares), *atm*-/- (darkly outlined open squares), *atm*-/-; *p53*+/- (open diamonds), and *atm*-/-; *p53*-/- (closed diamonds) mice following 8 Gy irradiation.

Fig. 4B is a photograph of the small intestine of a wild-type mouse at 20X magnification 4 days following 8 Gy irradiation.

Fig. 4C is a photograph of the small intestine of an *atm*-/- mouse at 20X magnification 4 days following 8 Gy irradiation.

Fig. 4D is a photograph of the small intestine of an *atm*-/-; *p53*-/- mouse at 20X magnification 4 days following 8 Gy irradiation.

Fig. 5 is a graph showing a mortality curve of mice following 10 Gy gamma irradiation. All of the p53 null mice (lightly outlined open squares), one-half of the p53 heterozygotic mice (closed diamonds), and no p53 wild-type mice (darkly outlined open squares) survived this irradiation dose.

Figs. 6A-6D are a series of photographs showing hematologic precursors eight days after irradiation in p53 wild-type and 53 null mice. In both in bone marrow (Fig. 6A, 10X magnification) and spleen (Fig. 6C, 5X magnification) of p53 wild-type mice, there was an absence of hematologic precursors following irradiation. In sharp contrast, p53 null mice showed a relative sparing of hematologic precursors in both bone marrow (Fig. 6B, 10X magnification) and spleen (Fig. 6D, 5X magnification).

Figs. 7A-7C are a series of graphs showing the comparison of various blood cell counts eight days after 10 Gy gamma irradiation (error bars indicated). Figure 7A shows that p53 null (KO) white blood cells, but not the white blood cells of wild-type (WT) or p53+/- (Het)mice, were found to be radioresistant to 10 Gy gamma irradiation. Figure 7B indicates that p53 null platelets, but not the platelets of wild-type or p53+/- mice, were also radioresistant. Figure 7C demonstrates the increased sparing of hematocrit after irradiation in p53 null mice as compared to the hematocrit of wild-type and p53+/- mice,

Fig. 8 is a graph showing the results of an *in vitro* hematopoietic colony

forming assay performed following gamma irradiation. Fitted linear-quadratic radiation survival curves are shown for *in vitro* hematopoietic colony forming cells (CFC) in wild-type (open squares), p53 null (darkly outlined open squares), atm null (closed diamonds), and atm/p53 double null (open diamonds) mice. Data points shown are the mean plus/minus standard errors for three to five individual mice per point. Loss of atm (see bottom two curves (*i.e.*, open and closed diamonds)) radiosensitized even p53 null bone marrow cells.

Fig. 9 is a graph showing the results of a mouse embryonic fibroblast (MEF) clonogenic survival assay. p53 null MEFs (upper curve, open squares) were resistant to the irradiation doses employed, while atm/p53 double null MEFs (lower curve, closed diamonds) were radiosensitive. Each curve represents the mean value per point, plus/minus the standard error.

Detailed Description

The invention is based upon studies in mice which did not express P53, did not express ATM, or did not express both P53 and ATM. Mice doubly null for ATM and P53 exhibited a dramatic acceleration of tumor formation relative to singly null mice, indicating that both genes collaborated in a significant manner to prevent tumorigenesis. With respect to their roles in apoptosis, loss of ATM rendered thymocytes only partially resistant to irradiation-induced apoptosis, whereas additional loss of P53 engendered complete resistance. This implied that the irradiation-induced ATM and P53 apoptotic pathways are not completely congruent. In contrast to prior predictions (Meyn *et al.*, Canc. Res. 55: 5991-6001,1995; Enoch and Norbury, Trends Biochem. Sci. 20: 426-430, 1995), ATM and P53 did not appear to interact in acute radiation toxicity, suggesting a separate ATM effector pathway for this DNA damage response.

Finally, we have shown that loss of atm leads to the p53-independent radiosensitization of multiple tissues. These discoveries enable methods and reagents for diagnosing and treating cancer of a variety of tissue types.

ATM, P53 double null mice are resistant to γ-irradiation induced thymocyte apoptosis, but remain sensitive to dexamethasone induced thymocyte apoptosis

Mutations in ATM and P53 cause the human cancer-associated diseases Ataxia-telangiectasia (Savitsky *et al.*, Science 268: 1749-1753, 1995) and Li-Fraumeni syndrome (Srivastava *et al.*, Nature 348: 747-749, 190; Malkin *et al.*, Science 250: 1233-1238, 1990), respectively. The most striking clinical finding in Ataxia-telangiectasia is an exquisite sensitivity to gamma irradiation. By contrast, loss of p53 in Li-Fraumeni disease is marked by radiation resistance in multiple tissue compartments.

ATM null mice (*i.e.*, *atm-/-*, Xu and Baltimore, Gen. Dev. 10: 2401-2410, 1996; Elson *et al.*, Proc. Natl. Acad. Sci. USA 93: 13084-13089, 1996; Barlow *et al.*, Cell 86: 159-171, 1996), as well as those null for P53 (*i.e.*, *p53-/-*, Donehower *et al.*, *supra*; Jacks *et al.*, *supra*), have been found to develop mainly T cell lymphomas, indicating that these genes have similar roles in thymocyte development. In order to study the interactions of these two genes on an organismal level, mice heterozygous for null alleles of both ATM and P53 were bred to produce all genotypic combinations of wild-type and mutant genes (Westphal *et al.*, Canc. Res 57: 1664-1667, 1997). Mice were generated by crossing P53 null mice in an FVB background to ATM null mice (Elson *et al.*, *supra*). F1 compound heterozygotes were crossed to generate all possible genotypic combinations.

atm-/-; *p53-/-* thymocytes were irradiated with 5 Gy, and apoptotic cell death was measured by propidium iodide uptake and cell shrinkage via flow

cytometry 0, 8, and 24 hours thereafter. Flow cytometry analysis (FACS) was performed on thymocyte samples obtained from 3 to 4 week old mice. Thymocytes were prepared from freshly prepared organs and plated in duplicates for control and treatment (irradiation or dexamethasone) time points. Flow cytometry was carried out on a FACScan (Becton Dickinson, San Jose, CA) using CellQuest software, and apoptotic cells were determined by propidium iodide staining at 0, 8, and 24 hours following either no treatment (control group), treatment with 5 Gy irradiation (irradiated group), or treatment with 1 μM dexamethasone (dexamethasone group). Three double null mice, three wild-type mice, and four *atm*+/+; *p53*-/- mice were analyzed, with each time point measured in duplicate. All mice were processed in parallel, and apoptosis was normalized using unirradiated control thymocytes at the given time points.

We found that double null thymocytes behaved as P53 null thymocytes, manifesting a similar complete resistance to gamma irradiation-induced apoptosis, as is shown in Fig. 1A. The profound resistance of double null thymocytes, as compared to the partial resistance of ATM null thymocytes, led to a model in which P53 modulates both ATM-dependent and ATM-independent apoptotic signals after ionizing radiation (see Fig. 2B).

Double null thymocytes were, however, sensitive to dexamethasone-induced apoptosis. In a parallel experiment, the results of which are shown on Fig. 1B, thymocytes of different genotypes were treated with the steroid, dexamethasone. Apoptosis was quantitated by FACS at 0, 8, and 24 hours. Shown on Fig. 1B is a comparison of the sensitivity to 1 μM dexamethasone of double null, P53 null, ATM null, and wild-type control thymocytes. As demonstrated in Fig. 1B, all genotypes manifested similar time-dependent apoptosis, with greater than 90% of cells dying by 24 hours, irrespective of

genotype, in response to 1 μM dexamethasone treatment, indicating that neither ATM nor P53 appeared to play a role in the dexamethasone-induced thymocyte cell death pathway.

ATM null thymocytes show delayed induction of P53 after irradiation

In order to understand the partial resistance of ATM null primary thymocytes to irradiation-inducing apoptosis in greater mechanistic detail, we assessed the induction of P53 protein in genetically defined primary thymocytes following gamma irradiation. Thymocytes were isolated from 3 to 4 week old mice and irradiated with 5 Gy from a Cs-137 irradiation source (Gammacell 40, Atomic Energy of Canada, Ltd.) at a dose rate of 1 Gy/min. Thymocytes were harvested at 1,2,4, and 6 hours after irradiation. Unirradiated control thymocytes were harvested in parallel with the 1 hour time point (labelled U in Fig. 2A). For each sample, $1x10^7$ cells were directly lysed in 1X SDS-PAGE loading buffer. Lysates corresponding to $5x10^6$ thymocytes were electrophoretically resolved on a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was probed with a mixture of pAB421 and pAB240 (Oncogene Sciences), and immunoreactive bands were detected using a horseradish peroxidase-coupled secondary antibody (Cappel) and enhanced chemiluminescence. Shown on Fig. 2A is a representative experiment demonstrating that irradiated ATM null thymocytes showed delayed induction of P53. Similar results were obtained from three additional ATM null mice and one additional wild-type mouse. As shown in Fig. 2A, maximal induction of P53 protein occurred about 2 hours after irradiation of wild-type thymocytes, whereas maximal induction of P53 was delayed to 4 to 6 hours in ATM null thymocytes. The delayed induction of P53 in ATM null primary thymocytes (Fig. 2A), and the partial resistance of ATM null

thymocytes to irradiation (Fig. 1A), suggested that ATM is involved in radiation-induced thymocyte apoptosis in a P53-dependent manner. However, these results clearly indicated that there is also an ATM-independent pathway for the induction of P53 protein and P53-dependent apoptosis after gamma irradiation, as is portrayed in the model schematically diagramed on Fig. 2B.

atm-/-; p53-/- and *atm-/-; p53+/-* mice exhibit accelerated tumorigenesis in comparison to *atm-/-; p53+/+* mice

Since ATM-independent activation of P53 and apoptosis was seen in irradiated thymocytes *in vitro*, we next determined if additional loss of P53 in an ATM null background altered tumorigenesis. Humans with Ataxia telangiectasia (Lavin and Shiloh, Ann. Rev. Immunol. 14: 177-202, 1996) and Li-Fraumeni syndrome (Srivastava *et al.*, *supra*; Malkin *et al.*, *supra*) are at markedly increased risk of tumorigenesis. Homozygous deletion of either ATM or P53 in mice leads predominantly to T cell lymphomas, which are first observed roughly at 3 months of age (Xu and Baltimore, *supra*; Elson *et al.*, *supra*; Barlow *et al.*, *supra*; Donehower *et al.*, *supra*; Jacks *et al.*, *supra*). We tested the possibility that ATM and P53 may have non-overlapping tumor-suppressing functions by looking for acceleration of tumor development in mice lacking both genes.

The survival data depicted on Fig. 3A represent thirteen ATM null mice; thirteen *atm+/-; p53-/-* mice; fifteen *atm-/-; p53+/-* mice; and eight double null mice. Lymphoid tumors were analyzed for the following cell surface markers: B220, IgM, CD4, CD8, Thy1, and CD3. Mice were observed until moribund and were subsequently euthanized. As shown on the mortality curve in Fig. 3A, 88% of ATM null mice survived past 100 days of age. A similarly large fraction (75%) of *atm+/-; p53-/-* mice also survived past 100 days of age. In

contrast, loss of P53 was associated with accelerated tumorigenesis in ATM null mice. None of the double null mice survived past 70 days. Moreover, more than half of the *atm*-/-; *p53*+/- mice died of cancer by 70 days, with only roughly 10% surviving past 100 days of age.

Histology of tumors arising in *atm*-/-; *p53*+/- mice was performed according to the following procedure: Mouse tissue was removed and fixed in Optimal Fix (American Histology Reagent, Lodi, CA), blocked in paraffin, sectioned at 10 μmeters, and stained with hematoxylin and eosin. Thirteen of the fourteen tumors in *atm*-/-; *p53*+/- mice were T cell lymphomas, as diagnosed by a combination of histology and flow cytometry. Similarly, most of the tumors seen in the ATM null state were T cell lymphomas (see Table 1 below).

Fig. 3B shows a representative Southern blot analysis of tumors which arose in *atm*-/-; *p53*+/- mice which indicated that the wild-type allele of *p53* was lost in three of seven tumors analyzed: the loss of *p53* heterozygosity seen in tumors in three of seven *atm*-/-; *p53*+/- mice tested (tail and tumor DNA from mice 1262 and 1292 are shown in bold), but not in four other mice (mouse 1249 is shown) is shown. Point mutations or functional inactivation of the remaining *p53* allele may have occurred in the other tumors. Thus, stochastic loss of the second *p53* allele or P53 haploinsufficiency can be associated with accelerated tumorigenesis, virtually exclusively in thymocytes.

Tumor types associated with various genotypes

Interestingly, as noted above and shown on Fig. 3A, every *atm*-/-; *p53*-/- mouse died before 70 days of age (n=8). As shown in Table 1, while *atm*-/-; *p53*+/+ (n=13) mice died exclusively of T cell lymphomas, the tumor spectrum of double null mice appeared altered.

Table I

Tumor Types Associated with Various Genotypes

Genotype	Age at Death	number	Tumor Type
atm-/- p53+/+	85-100 days	2	T cell lymphomas
	>100 days	11	T cell lymphomas
atm-/- p53+/-	Avg. of 70 days	13	T cell lymphomas
		1	teratoma
		1	unknown
atm+/-; p53-/-	>100 days	5	no pathology to date
	124 days	1	thymic lymphoma and sarcoma
	124 days	1	sarcoma
	120 days	1	thymic lymphoma and sarcoma
	91 days	1	thymic lymphoma
	85 days	1	thymic lymphoma and sarcoma
	63 days	1	thymic lymphoma
	49 days	1	teratoma
atm-/-; p53-/-	70 days	1	teratoma
	69 days	1	T cell lymphoma and sarcoma
	61 days	1	cause of death unknown
	57 days	1	T cell lymphoma and sarcoma
	49 days	1	B cell lymphoma
	45 days	1	lymphoma devoid of T/B markers
	44 days	1	sarcoma
	38 days	1	cause of death unknown

All *atm-/-*mice, and thirteen out of fourteen *atm-/-; p53+/-* mice died of T cell lymphomas. *atm-/-* mice generally survived past 100 days of age, while *atm-/-; p53+/-* mice died at an average age of 70 days. By contrast, *atm+/-; p53-/-* mice died of either T cell lymphoma or sarcoma, at an average of 90 days of age. Exhibiting accelerated tumorigenesis, *atm-/-; p53-/-* mice died at an average of 54 days of age. *atm-/-; p53-/-* mice succumbed to B cell

lymphomas and lymphomas lacking T and B cell markers, in addition to the T cell lymphomas and sarcomas seen in mice with other genotypic combinations. One B cell lymphoma (a type not previously reported in P53 and ATM single null mice) was observed, and another lymphoma was negative for both T and B cell markers. In addition, sarcomas and a teratoma were seen in these mice. Taken together, these data demonstrated an unexpected broadening of the tumor spectrum in double null mice. Interestingly, homozygous deletion of *atm* in the P53 null background appeared to accelerate P53-spectrum tumors such as sarcomas and teratomas (compare age at incidence of tumors seen in *atm*+/-; *p53*-/- mice to that seen in double null mice in Table 1).

Increased acute radiation sensitivity in ATM null mice

One of the most striking and defining characteristics of Ataxia telangiectasia is acute radiation sensitivity, with radiation dermatitis and ulcerations of the gastrointestinal tract after therapeutic irradiation (Gotoff *et al.*, Amer. J. Dis. Child. 114: 617-625, 1967). Since we had observed interactions between ATM and P53 in apoptosis and tumorigenesis, an analysis was made to determine if these two genes interacted in acute radiation toxicity.

Four week old (27-33 day old) wild-type, P53 null, ATM null, ATM null/P53 heterozygotic, and double null mice were irradiated with 8 Gy at a dose of 122 cGy/minute with a Cs-137 irradiation source (Mark 1 Irradiator, J.L. Shepherd & Sons, San Bernardino, CA). Mice were subsequently housed together, fed *ad libitum*, and checked once daily for clinical status. In total, eight wild-type, three *p53*-/-, five *atm*-/-, fifteen *atm*-/-; *p53*+/-, and four *atm*-/-; *p53*-/- mice were irradiated.

As shown in Fig. 4A, ATM and P53 did not appear to interact in mediating acute irradiation sensitivity. Wild-type and P53 null mice did not

rapidly succumb to 8 Gy irradiation. Only one out of the eight wild-type mice died with delayed kinetics 2 weeks after this dose, while no *p53-/-* mouse died. In sharp contrast, *atm-/-*; *p53+/-*, *atm-/-*, and *atm-/-*; *p53-/-* mice manifested similar acute sensitivity to 8 Gy irradiation; hence, all *atm-/-* mice, irrespective of P53 status, died rapidly, exhibiting diarrhea and malaise as early as 3 days, and loss of hair by 4 days after irradiation. ATM null mice generally died between 5 and 8 days after irradiation. This time frame is consistent with gastrointestinal toxicity. In order to confirm the suspected cause of death and document differences in radiation sensitivity between mice of different genotypes, lymphoid organs, gastrointestinal tract, and skin were analyzed pathologically in mice 4 days after 8 Gy irradiation.

As shown in Fig. 4B, the small intestine (magnified at 20X) of the wild-type mouse showed no significant changes in the gastrointestinal tract four days following 8 Gy irradiation. The irradiated wild-type mouse had normal-appearing intestinal villi and muscularis mucosa. In contrast, both *atm-/-* (Fig. 4C) and *atm-/-*; *p53-/-* (Fig. 4D) mice were afflicted with acute radiation enteritis 4 days after 8 Gy irradiation, both also shown at 20X magnification. The normal villus architecture was severely disrupted by edematous changes. In addition, the large and small intestine of ATM null (Fig. 4C) and double null (Fig. 4D) mice was marked by severe edema and destruction of normal architecture (especially severe in the small intestine, shown at 20X in Figs. 4C and 4D), while the lymphoid system of the ATM null mouse was depleted as in the wild-type animal. In addition, the skin of ATM null and double null mice showed edematous changes not seen in the wild-type mouse. Other organs appeared normal. The acute skin changes and radiation enteritis seen mirrored the observations made in humans with Ataxia telangiectasia (Gotoff *et al.*, *supra*; Cunliffe *et al.*, Brit. J. Rad 48: 374-376,

1975; Morgan *et al.*, Amer J. Dis. Child. 116: 557-559, 1968). Since this acute radiation toxicity appeared unaltered by loss of P53, we determined that P53 is unlikely to play a role in the acute radiation toxicity seen in ATM null humans and mice.

Loss of atm radiosensitizes multiple p53 null tissues

Previous studies have shown that, *in vitro*, p53-deficient bone marrow cells are resistant to gamma irradiation. Since we have demonstrated that the gastrointestinal radiosensitization engendered by loss of atm is independent of p53, we next looked at *in vivo* bone marrow resistance to gamma irradiation in p53 deficient mice.

During the irradiation studies described above, we observed differences in p53 null versus wild-type mouse *in vivo* irradiation sensitivity (see, *e.g.*, Fig. 4A). In order to address this issue in a more rigorous manner, we compared inbred FVB strain mice differing only in their p53 genotype. p53 null mice, first described in Donehower *et al.* (Nature 356: 215-221, 1992), were obtained from Larry Donehower (Baylor College of Medicine, Houston, TX) and backcrossed ten generations into a pure FVB background. Mice were irradiated with 10 Gy in a Cs-137 irradiator at a dose rate of 1.2 Gy per minute, and were scored for survival up to 30 days. As illustrated in Fig. 5, we found that survival after irradiation was dependent upon p53 genotype in a dose-dependent manner. After 10 Gy irradiation, all wild-type mice, but only roughly half of p53 heterozygotic mice and no p53 null mice, died (Fig. 5).

The time course of death, namely one to two weeks after irradiation, was consistent with acute bone marrow toxicity. We hence performed pathological analyses in order to address this hypothesis (Figs. 6A-6D). In order to determine the cause of death, mice of various genotypes were sacrificed eight

days after 10 Gy irradiation and tissues was analyzed pathologically as described above. A striking sparing (*i.e.*, lack of death) of hematopoietic precursor cells was seen in p53 null (Fig. 6B) versus wild-type (Fig. 6A) bone marrow. Furthermore, an analysis of splenic tissues revealed similarly spared p53 null (Fig. 6D) versus wild-type (Fig. 6C) hematologic precursors. Pathological data were, hence, consistent with an increased *in vivo* resistance of p53 null hematologic tissues to gamma irradiation.

In order to confirm these observations, we performed complete blood counts of mice eight days after 10 Gy gamma irradiation (Figs. 7A-7C). Blood samples were obtained by tail bleed in tandem, and complete blood counts were performed. These data confirmed that, *in vivo*, white blood cells (Fig. 7A) and platelets (Fig. 7B) are made radioresistant by deletion of p53. The relative sparing of hematocrit seen in p53 null mice (Fig. 7C) further supports this notion. The data shown in Figs. 5, 6A-6D and 7A-7C hence argues that loss of p53 engenders *in vivo* radioresistance, via protective effects on blood cell precursors.

Interactions between atm and p53 control cellular proliferation mediate the G1/S cell cycle checkpoint, regulate thymocyte apoptosis, and modulate tumorigenesis. In contrast to previous hypotheses (Meyn *et al.*, Canc. Res. 55: 5991-6001,1995; Enoch and Norbury, Trends Biochem. Sci. 20: 426-430, 1995), we have demonstrated above that atm and p53 do not interact in the acute radiation response of gastrointestinal tissues. Instead, we found that loss of atm radiosensitizes even p53 null gastrointestinal tissues. We next determined how atm and p53 interact in the radiation response in bone marrow cells in an *in vitro* hematopoietic colony forming cell assay.

To performed the *in vitro* hematopoietic colony forming assay, bone marrow was harvested from both femora of individual mice and resuspended in

Fischers medium. The suspension was counted to give the number of cells per femur and then divided into four portions for irradiation with 0, 2, 4 or 6 Gy in a Cs-137 irradiator (dose rate was approximately 3.5 Gy/minute). Immediately after irradiation, *in vitro* hematopoietic colony forming cells (CFC) were cultured as described previously (Heyworth and Spooncer, "In vitro clonal assays for murine multipotential and lineage-restricted progenitor cells." In: Haemopoiesis: A Practical Approach, eds. N.G. Testa and G. Molineux, pp. 37-54, Oxford University Press, 1993). Briefly, appropriate volumes of bone marrow suspension were resuspended in Fischers medium supplemented with 20% fetal calf serum, 0.33% agar, and recombinant murine IL-3. One mL of the culture mixture was placed in each of three 35 mm petri dishes and incubated in a humidified incubator at 37°C, with 5% O_2 and 5% CO_2. Granulocyte/macrophage colonies were counted after 7 days of growth using a stereo microscope. Cell numbers required to generate adequate colony counts at each radiation dose were estimated from historical cell survival fractions for BDF1 mice, as described previously (Wang *et al.*, Radiat. Res. 146: 259-266, 1996). Surviving fractions of irradiated cells to non-irradiated cells were 1.0 (*i.e.*, 100%) at 0Gy, 0.5 (*i.e.*, 50%) at 2Gy, 0.16 (*i.e.*, 16%) at 4Gy, and 0.04 (*i.e.*, 4%) at 6Gy.

Consistent with previous *in vitro* studies (Cui *et al.*, J. Environ. Pathol. Toxicol. Oncol. 14: 159-163, 1995), we found that loss of p53 rendered bone marrow cells radioresistant *in vitro* (Fig. 8, darkly outlined squares, top curve). In sharp contrast, loss of atm radiosensitized bone marrow cells (Fig. 8, closed diamonds, bottom curve). Strikingly, the atm null radiosensitization was present even in p53 null bone marrow cells, such that atm null and atm/p53 double null bone marrow cells were significantly more sensitive to irradiation than wild-type cells (Fig. 8, open and closed diamonds, bottom curves). Hence,

we have demonstrated that p53 null mice manifest *in vivo* resistance of hematologic precursor cells to gamma irradiation. Furthermore, our data showed that a loss of atm is dominant to loss of p53 in radiosensitizing bone marrow cells.

We next analyzed the interactions between atm and p53 in fibroblasts. MEFs were derived using standard procedures (Deng *et al.*, Cell 82: 675-684, 1995). Briefly, day 11.5 to 16.1 post coitum embryos were dissociated, treated with DNase and trypsin, and plated in DMEM containing 15% fetal calf serum. Exponentially growing MEFs were seeded (300 cells per well on six-well tissue culture plates) and then irradiated with the indicated doses on the following day. After 1 week, the colonies were fixed in cold methanol and stained with trypan blue. Each experiment was performed in triplicate for each independent experiment. Cloning efficiencies (termed the surviving fraction) were calculated as the number of colonies divided by the number of cells seeded for each treatment, normalized to the control (unirradiated) plating efficiency. A Cs-137 irradiation source at a dose rate of 85 cGy per minute was used. In a comparison of the radiation response in atm/p53 double null (closed diamonds) mouse embryonic fibroblasts (MEFs) versus p53 null (open squares) MEFs, loss of atm was found to radiosensitize p53 null cells (Fig. 9).

In summary, we have demonstrated that ATM and P53 interact in a complex manner *in vivo*. Our data indicated that multiple p53 null tissues (*i.e.*, gastrointestinal cells, bone marrow cells, and fibroblasts), are radiosensitized by loss of atm; hence, loss of atm leads to p53-independent radiosensitization. Since atm is a protein kinase (Keegan *et al.*, Genes Dev. 10: 2423-2437, 1996), functional inhibition of this protein is valuable as an adjunct to gamma irradiation or other DNA damaging therapies in the treatment of both p53 null and p53 wild-type human tumors in a variety of tissues. Functional

inactivation of ATM may sensitize tumor cells to therapeutic irradiation in a manner that is independent of P53 status. Such an approach would have to be carefully tailored to susceptible tumor subtypes, and focused to overcome toxicity in surrounding tissues.

Our studies have highlighted several intriguing features of the interactions between ATM and P53. ATM loss may either sensitize (in the case of gut epithelium) or protect (in the case of thymocyte apoptosis) cells from ionizing radiation. This paradoxical behavior appeared to be cell type-specific and could reflect differences in the molecular determinants of the apoptotic threshold (Fisher, Cell 78: 539-543, 1994). However, the delayed but remaining induction of P53 protein and the partial apoptotic response observed in ATM null thymocytes clearly demonstrated the existence of an alternative radiation-induced pathway involving P53 that functions in the absence of ATM. Similar signals appeared to operate during tumorigenesis, as additional P53 loss can be identified in *atm*-/-; *p53*+/- mice. In addition, ATM/P53 double null mice exhibited accelerated tumorigenesis, with a broadening of the tumor spectrum. Since loss of ATM and P53 appeared not to be functionally equivalent and their simultaneous loss substantially accelerated tumorigenesis, these studies suggested the potential importance of examining human neoplasms for the status of both genes.

Polypeptide fragments of ATM family members

Patients with ataxia telangiectasia (AT) share with Li-Fraumeni syndrome patients a propensity to develop thymic lymphomas. The *atm* gene was identified by positional cloning and genome analysis of samples taken from AT patients (Savitsky *et al.*, Science 268: 1749-1753, 1995). The *atm* gene product was found to be a nuclear phosphoprotein with associated protein

kinase activity (Chen and Lee, J. Biol. Chem. 271: 33693-33697, 1996; Jung *et al.*, Canc. Res. 57: 24-27, 1997; Xu and Baltimore, *supra*). The ATM protein is a member of the four member family of Phosphatidyl inositol-3 (PI3) kinase-related protein kinases, a family which is also referred to as the ATM protein kinase family (Hoekstra, M.F., Curr. Opin. Genet. Dev. 7: 170-175, 1997). Although the four members of this family, ATM, ATR, FRAP, and DNA-PK, share sequence similarity to the p110 lipid kinase subunit of PI-3 kinase, none has yet been demonstrated to have PI-3 kinase activity. However, the four ATM protein kinase family members all have a carboxy-terminal protein kinase domain, and have been found to be associated with a protein kinase activity (Hoekstra, *supra*).

Polypeptide fragments of ATM protein kinase family members are useful in blocking biological activities of these protein kinases. Methods for generating such fragments are well known in the art (see, for example, Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1994); such fragments are made on the basis of the published nucleotide sequence of the genes encoding the various members of the family: *atm* (Savitsky *et al.*, Science 268: 1749-1753, 1995; Savitsky *et al.*, Hum. Mol. Genet. 4: 2025-2032, 1995), *atr* (Bentley *et al.*, EMBO J. 15: 6641-6651, 1996), *frap* (Brown *et al.*, Nature 369: 12574-12578, 1994; Chiu *et al.*, Proc. Natl. Acad. Sci. USA 91: 12574-12578, 1994; Sabatini *et al.*, Cell 78: 35-43, 1994; Sabers *et al.*, J. Biol. Chem. 270: 815-822), and *dna-pk* (Hartley *et al.*, Cell 82: 849-856, 1995). For example, an ATM polypeptide fragment may be generated by PCR amplification of the corresponding nucleotide fragment using oligonucleotide primers based upon the *atm* nucleic acid sequence. Preferably, the oligonucleotide primers bear unique restriction enzyme sites which facilitate insertion of the nucleotide fragment into the cloning site of a

mammalian expression vector. This vector may then be introduced into a mammalian cell by any of the standard techniques known in the art (*i.e.*, transfection by, *e.g.*, DEAE-dextran, electroporation, or $CaPO_4$ precipitation), for production of the desired recombinant ATM polypeptide fragment.

Polypeptide fragments of ATM family members may be used to reduce biological functions of the full length proteins by inhibiting binding of the full length proteins to their substrates. For example, ATM has been found to phosphorylate I-κB-α (Jung *et al.*, *supra*). Hence, an ATM polypeptide fragment may be generated which blocks binding of endogenous full-length ATM protein to I-κB-α, thus preventing I-κB-α phosphorylation. Such an ATM polypeptide fragment may consist, for example, of an I-κB-α binding site which lacks the C-terminal protein kinase effector domain.

Polypeptide fragments of ATM protein kinase family members may be expressed as recombinant proteins using methods known in the art and described herein. Alternatively, a mammalian expression vector containing DNA encoding an ATM family member polypeptide fragment may be introduced into a desired cell. The DNA encoding the fragment may be operably linked to any suitable promoter (*e.g.*, the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in lymphocytes or muscle cells may be used to direct the expression of an ATM protein kinase family member. The enhancers used may include, without limitation, those that are characterized as tissue- or cell-specific in their expression.

ATM family mutants which lack biological activity

The biological function of a member of the ATM protein kinase family

is a protein kinase activity. Mutant ATM family member proteins which lack this protein kinase activity are useful in blocking the biological activity of endogenous ATM protein kinase family members when overexpressed in the same cell. Such mutants may be generated by point mutation, deletion, or insertion, using techniques well known in the art (see, for example, Ausubel *et al.*, *supra*).

Bacterial cloning plasmids, such as the pUC series of plasmids commercially available from Clontech, are preferred for the generation of ATM mutants and fragments because they are, in general, small, easily manipulated, and maintained at a high copy number in a transformed bacterium. Another useful characteristic of bacterial cloning plasmids is that they have relatively few recognition sequences for restriction endonucleases, which allows for the utilization of restriction endonuclease recognition sequences internal to the insert which, in this case, is DNA encoding the ATM family member polypeptide.

Such a mutant may be generated in a bacterial plasmid, followed by sequence analysis by standard techniques. DNA encoding a desired mutant may then be subcloned into a mammalian expression vector.

Given the relatively large sizes of the four ATM family members (all are greater than 2500 amino acids in length), it may be desirable to generate nucleic acid mutants in a fragment of DNA carried on a bacterial plasmid, for example, a fragment consisting of the C-terminal protein kinase domain. Following mutagenesis and sequence analysis, the mutant fragment may then be subcloned into a mammalian expression vector with the remaining, un-mutated portion of the full length protein to produce a DNA that encodes for full length mutant protein.

Once subcloned into a mammalian expression vector, the mutant protein

may be produced recombinantly *in vitro*, or may be produced *in vivo* in a desired cell introduced with the mammalian expression vector encoding the mutant protein.

ATM family member-specific neutralizing antibodies

Both polyclonal and monoclonal neutralizing antibodies which specifically recognize ATM, ATR, FRAP, or DNA-PK polypeptides may be generated using methods well known in the art (see, for example, Kohler *et al.*, Nature 256: 495, 1975; Kohler *et al.*, Eur. J. Immunol. 6: 511, 1976; Kohler *et al.*, Eur. J. Immunol. 6: 292, 1976; Hammerling *et al.*, In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, New York, NY, 1981; Ausubel *et al.*, *supra*). In order to prepare neutralizing antibodies which are specific toward, for example, ATM, ATM proteins, polypeptide fragments, or fusion proteins may be used as antigen. Resulting antisera and antibody-containing supernatants may be screened for ATM specificity by ELISA, Western blotting, and/or immunoprecipitation analysis (by the methods described in Ausubel *et al.*, *supra*).

Monoclonal and polyclonal antibodies that are neutralizing antibodies which block the protein kinase activities of ATM family members are useful in the invention. Antibodies which neutralize the protein kinase activity of, for example, ATM, may be identified by adding ATM-specific antibodies to an *in vitro* ATM kinase activity assay, such as the assay described by Jung *et al.* (*supra*). To assay for protein kinase activity of ATM, lysates from ATM-expressing cells are incubated with ATM-specific antibodies. Such antibodies may be generated according to standard techniques, and are also commercially available (from, *e.g.*, Santa Cruz Biotechnology). The bound proteins may then be precipitated with protein A sepharose coated beads, and washed to remove

any non-specifically associated proteins. Immunoprecipitated ATM proteins are then incubated for 15 minutes at room temperature in the ATM kinase buffer consisting of 20 mM Hepes-NaOH, 10 mM $MgCl_2$, and 10 mM $MnCl_2$ (pH 7.4) supplemented with 2.5 μCi γ-32P-labelled ATP, 50 μM ATP, and 1 μg of an ATM substrate, such as the recombinantly produced GST-IκB-α-C fusion protein described by Jung *et al.* (*supra*). ATM-specific antibodies being tested for an ATM protein kinase neutralizing ability may be added to this reaction mixture at varying concentrations, retaining as controls reaction mixtures which are not administered ATM-specific antibodies being tested for ATM neutralizing ability. The reaction mixture is then resolved by SDS-PAGE and subjected to autoradiography. An ATM-specific antibody with neutralizing ability is able to lower the amount of phosphorylation of GST-IκB-α-C when compared to a control reaction.

It will be understood by the skilled artisan that variations of the *in vitro* kinase reaction may be employed to detect neutralizing antibodies. For example, to detect an ATM neutralizing antibody, the substrate need not be GST-IκB-α-C, and the immunoprecipitated proteins need not be SDS-PAGE resolved prior to autoradiography. ATM neutralizing antibodies may also be screened in high-throughput assays. For example, ATM proteins may be immobilized by plate-bound ATM-specific antibodies, and the *in vitro* kinase assays conducted in the plate. In a multi-well plate, several different ATM-specific antibodies may be simultaneously screened for an ATM neutralizing ability. Phosphorylation of an ATM substrate may be assessed by harvesting well components onto porous filtermats such that unincorporated γ-32P-labelled ATP are not retained. The filtermat-bound ATM substrates may then be assessed for γ-32P-labelled ATP incorporation on a scintillation counter.

In addition to intact monoclonal and polyclonal neutralizing anti-ATM

antibodies, the invention can employ genetically engineered antibodies, humanized antibodies, and antibody fragments, including F(ab')2, Fab', Fab, Fv, and sFv fragments. Antibodies can be humanized by methods known in the art, *e.g.*, monoclonal antibodies with a desired binding specificity can be commercially humanized (Scotgene, Scotland; Oxford Molecular, Palo Alto, CA). Fully human antibodies, such as those expressed in transgenic animals, are also features of the invention (Green *et al.*, Nature Genetics 7:13-21, 1994).

Ladner (U.S. Patent 4,946,778 and 4,704,692) describes methods for preparing single polypeptide chain antibodies. Ward *et al.* (Nature 341:544-546, 1989) describe the preparation of heavy chain variable domains, which they term "single domain antibodies," which have high antigen-binding affinities. McCafferty *et al.* (Nature 348:552-554, 1990) show that complete antibody V domains can be displayed on the surface of fd bacteriophage, that the phage bind specifically to antigen, and that rare phage (one in a million) can be isolated after affinity chromatography. Boss *et al.* (U.S. Patent 4,816,397) describe various methods for producing immunoglobulins, and immunologically functional fragments thereof, which include at least the variable domains of the heavy and light chain in a single host cell. Cabilly *et al.* (U.S. Patent 4,816,567) describe methods for preparing chimeric antibodies.

Antisense nucleic acid

Another potential reagent capable of blocking the biological activities of ATM protein kinase family members is antisense nucleic acid corresponding to DNA encoding ATM protein kinase family members. The principle is based upon the hypothesis that sequence-specific suppression of gene expression can be achieved by intracellular hybridization between mRNA encoding a ATM

family member and a complementary antisense species. The formation of a hybrid RNA duplex may then interfere with the processing, transport, translation, and/or stability of the targeted mRNA. Antisense strategies may use a variety of approaches including the use of antisense oligonucleotides and injection of antisense RNA. The antisense mRNA may be produced and isolated by any standard technique, but is most readily produced by *in vitro* transcription using an antisense cDNA under the control of a high efficiency promoter (*e.g.*, the T7 promoter). Administration of antisense mRNA to cells may be carried out by any of the methods for direct nucleic acid administration. Transfection of antisense RNA expression vectors into targeted cells may also be employed. Phenotypic effects induced by antisense nucleic acid are based on changes in criteria such as protein levels, protein kinase activity measurement, and target mRNA levels.

In vitro screens for compounds which reduce biological functions of ATM family members

A tumor with reduced levels of P53 biological function may be subjected to a variety of manipulations both *in vivo* and *in vitro* to induce a reduction in the biological activity of the gene products from ATM family member genes. Compounds, such as small chemical compounds, being tested for an ability to reduce the biological function of one or more members of the ATM protein kinase family may be screened by a high-throughput assay for protein kinase activity, such as the ATM protein kinase assay described herein. It is understood that a compound that specifically blocks, for example, FRAP biological activity may be used in combination with a compound that blocks the biological activity of another ATM protein kinase family member. In addition, compounds which are found to specifically block more than one

member of the family are useful in the invention and may be administered to a target P53 non-expressing cell.

In vivo screens for compounds which reduce biological functions of ATM family members

Compounds, whether identified using *in vitro* screens, or whether a neutralizing antibody, a mutant protein, a polypeptide fragment, or an antisense nucleic acid species, can be further tested for an ability to reduce the biological function of ATM family members by an *in vivo* screen. Compounds may also be screened *de novo* in this *in vivo* screen. To conduct the screen, tumors from ATM non-expressing mice (ATM null), and tumors from mice which do not express either ATM or P53 (ATM null; P53 null) are implanted into a-thymic nude mice. Compounds are then administered to the nude mice implanted with P53 null tumors, retaining untreated control P53 null cancerous nude mice. The compounds may be administered by various methods, as described herein, at various concentrations, and at various times (*e.g.*, prior to implantation, following implantation, repeated administrations). Tumor implanted nude mice are then subjected to whole body irradiation with 8 Gy. A compound which blocks the biological activity of an ATM family member will cause the tumors in treated P53 null cancerous nude mice to respond to radiation toxicity more rapidly following irradiation than untreated P53 null cancerous nude mice. As a positive control, ATM null; P53 null tumors in nude mice will deteriorate rapidly (*i.e.*, within 7 days) following irradiation. Such deterioration due to radiation toxicity may be assessed histologically using standard techniques.

To study the effects of a compound on a specific tumor type, transplanted tumors from P53 null and double null mice may be of various lineages, for example, of the thymic lymphoma lineage. With a site-localized

tumor, it may be desirable to administer local irradiation, instead of whole body irradiation. With either local or whole body irradiation, it is understood that a non-lethal dose of irradiation may also be administered, and the condition of the tumor graded histologically following irradiation.

Tumors from other sources may be used to implant a-thymic nude mice. Such tumors from mice, humans, or other mammals are known in the art and are also commercially available (from, for example, the American Type Culture Collection, Rockville, MD). These tumors may be assessed for P53 expression by, for example, Western blotting analysis or ELISA, with commercially available P53-specific antibodies (from, *e.g.*, Santa Cruz Biotechnology). These tumors implanted into nude mice may then be assessed for susceptibility to γ-irradiation with or without prior treatment with a compound being screened for an ability to reduce the biological activities of ATM family members. Preferably, a human tumor will be implanted, such that a compound capable of blocking the function of human ATM family members may be identified.

Administration of ATM family biological function reducing compounds

A compound that reduces the biological function of the *atm, atr, frap,* and/or *dna-pk* gene products may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer an ATM family member biological function reducing compound to patients with neoplasms.

Administration may begin before the patient is symptomatic. Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intra-arterial, subcutaneous,

intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, by suppositories, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in Remington's Pharmaceutical Sciences, (18th edition), ed. A. Gennaro, Mack Publishing Company, Easton, PA, 1990. Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

Dosage requirements for administration of compounds capable of reducing biological function of ATM family members

Dosage is determined by standard techniques and is dependent, for example, upon the weight of the patient and the type or extent of the neoplasm being treated. Dosage requirements may also be determined by administering an ATM family member biological function-reducing compounds to animal

models. For example, one such animal model is the P53 null mouse (Donehower et al, *supra*). Mice deficient for P53 expression (P53 null) are characterized by an increased incidence of cancers, which are primarily thymic lymphomas (Jacks *et al.*, *supra*). A compound, or combinations thereof, that reduces biological function of one or more members of the ATM family of protein kinases may be administered globally to a cancerous P53 null mouse at varying concentrations. Histological examination of control and treated animals may then be performed to assess reduction in cancerous cells in treated versus untreated mice, and, in addition, assess any possible side effects of the compound(s) in the treated animals. A potentially therapeutic dose of the compound(s) will cause the greatest reduction in cancer cell number with the fewest averse side effects in the treated animal as compared to the untreated animal.

An alternative approach to determine dose requirements for a specific type of tumor is to inject murine P53 non-expressing tumor cells of various lineages (*e.g.*, a liver tumor cell) into syngeneic mice (or nude mice). One source for such a tumor is the P53 null mouse. Once the injected mouse has developed a tumor that is measurable (*e.g.*, with calipers), varying concentration of an ATM function-reducing compound, or combinations thereof, may be administered to the cancerous mouse. Again, an appropriate potentially therapeutically effective dose of compound will induce the greatest reduction in tumor size with the fewest averse side effects.

Anti-cancer therapies

The results described herein demonstrate that a reduction in the biological function of the *atm* gene product sensitized cancers with reduced levels of P53 function to the cell death-inducing effects of γ-irradiation. A

reduction in the expression of the other members of the ATM family is similarly capable of sensitizing cancers with reduced P53 expression to anti-cancer therapies.

a) Irradiation therapy

Prior to, concurrent with, or following administration of one or more compounds that reduce the biological activities of one or more of the ATM family members to a patient with a cancer characterized by a functionally reduced level of P53, the patient is treated with a therapeutically effective amount of γ-irradiation. The γ-irradiation therapy may be administered locally to the site of the tumor, or globally, as deemed appropriate. It will be understood that the order in which the cancer patient is administered irradiation therapy and the compound(s) is not necessarily pre-determined, and may vary on a case to case basis. Such administration strategies may be first tested in the *in vivo* models described herein. For example, the patient may receive alternating administrations of irradiation and compound(s). The patient may also receive just one administration of the compound(s), and a number of treatments with irradiation therapy. It is also understood that the patient may have received treatments with irradiation or other anti-cancer therapies prior to administration of the compound(s).

b) Other therapies

Various other anti-cancer therapies, including chemotherapies, are known in the art and may be used in combination with one or more compounds capable of reducing the biological activity of one or more of the members of the ATM protein kinase family to treat a patient with a cancer characterized by reduced P53 expression levels. Each type of therapy may first be tested in the various *in vitro* and *in vivo* assays described herein for a heightened effect on a cancer with reduced P53 expression and reduced expression of one or more of

the ATM protein kinase family members. For example, a human carcinoma biopsied from a patient may be implanted into a nude mouse with or without administration of a compound(s) capable of reducing expression of one or more of the ATM protein kinase family members. Following onset of a measurable amount of cancer, the implanted nude mice may then receive, for example, administration of a chemotherapeutic agent at varying concentrations. By this method, drug efficacy and dosage requirements may be rapidly assessed on a specific tumor type. The carcinoma bearing patient may then receive treatment strategies found to be most effective in the transplanted nude mice.

Detection of cancers which will favorably respond to γ-irradiation therapy

Given the rapid rate of growth and spread of cancer cells, it is often detrimental to a favorable outcome should the incorrect therapy be administered. Although the relative sensitivity of certain tumor types to certain therapies has been assessed (*e.g.*, fibrosarcomas do not respond well to irradiation therapies), the present invention allows for the rapid determination of the sensitivity of a cancer to irradiation therapy. A patient with cancer may have a biopsy to remove cancerous cells. The cancerous cells may then be assessed for a reduced level of expression of P53, ATM, ATR, FRAP, and DNA-PK. Expression levels may be determined by Western blot analysis of cell lysates with, for example, P53-specific antibodies, where immunoreactive bands may be visualized using non-radioactive chemiluminescent reagents. Expression levels may also be rapidly assessed in an ELISA-type assay with antibodies specific for the five proteins. The colorimetric reactivity may then be assessed on a microtiter plate reader. Protocols for these methods are well known in the art (see, for example, Ausubel *et al.*, *supra*). A tumor which has a reduced level of P53 in addition to a reduced level of at least one of the ATM

protein kinase family members will respond favorably to irradiation therapy. The dosage and site distribution (*i.e.*, global or local administration) may be determined using methods described herein.

All publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication was specifically and individually indicated to be incorporated by reference.

What is claimed is:

Claims

1. A method for treating a neoplasm characterized by a decreased level of functional P53 protein, said method comprising the steps of:

(a) inducing a decreased level of biological activity of an ATM protein in cells of said neoplasm to enhance the sensitivity of said neoplasm to irradiation; and

(b) exposing said neoplasm to a neoplastic cell killing amount of irradiation.

2. The method of claim 1, wherein said inducing is by introduction into said neoplasm of neutralizing antibody specific for an ATM protein.

3. The method of claim 1, wherein said inducing is by introduction into said neoplasm of an ATM polypeptide fragment or mutant capable of competitively inhibiting the biological activity of said ATM protein.

4. The method of claim 1, wherein said inducing is by introduction into said neoplasm of nucleic acid capable of expressing an ATM protein polypeptide fragment or mutant capable of competitively inhibiting the biological activity of said ATM protein.

5. The method of claim 1, wherein said inducing is by introduction into said neoplasm of antisense ATM nucleic acid which inhibits expression of an ATM protein.

6. The method of claim 1, wherein said neoplasm is derived from a bone marrow cell, a gastrointestinal cell, or a fibroblast.

7. A method for screening a compound for potential use as a anti-cancer therapeutic agent useful to treat cancers characterized by decreased levels of functional p53 protein, said method comprising the steps of:

(a) contacting said compound with an ATM protein, and

(b) determining whether said compound reduces a biological activity of said ATM protein, wherein the reduction of ATM protein biological activity by said compound indicates potential efficacy of said compound as an anti-cancer agent.

8. A method for determining whether a compound is capable of reducing the biological activity of an ATM protein, said method comprising the steps of:

(a) providing a mammal having a neoplasm characterized by reduced p53 expression;

(b) administering to said mammal said compound and anti-cancer therapy; and

(c) determining whether the therapeutic response of said mammal to said anti-cancer therapy is enhanced by said compound, compared to the response of said mammal to said therapy in the absence of said compound, an enhanced response indicating that said compound decreases the biological activity of said ATM protein.

9. The method of claim 8, wherein said mammal is a rodent.

10. The method of claim 8, wherein said mammal is immunocompromised.

11. The method of claim 8, wherein said anti-cancer therapy is administration of γ-irradiation or a chemotherapeutic anti-cancer agent.

12. A method for determining whether a neoplasm will favorably respond to irradiation, said method comprising measuring the level of expression or biological activity of an ATM protein in cells of said neoplasm, a level lower than the level in cells of a fibrosarcoma indicating probable favorable response to irradiation.

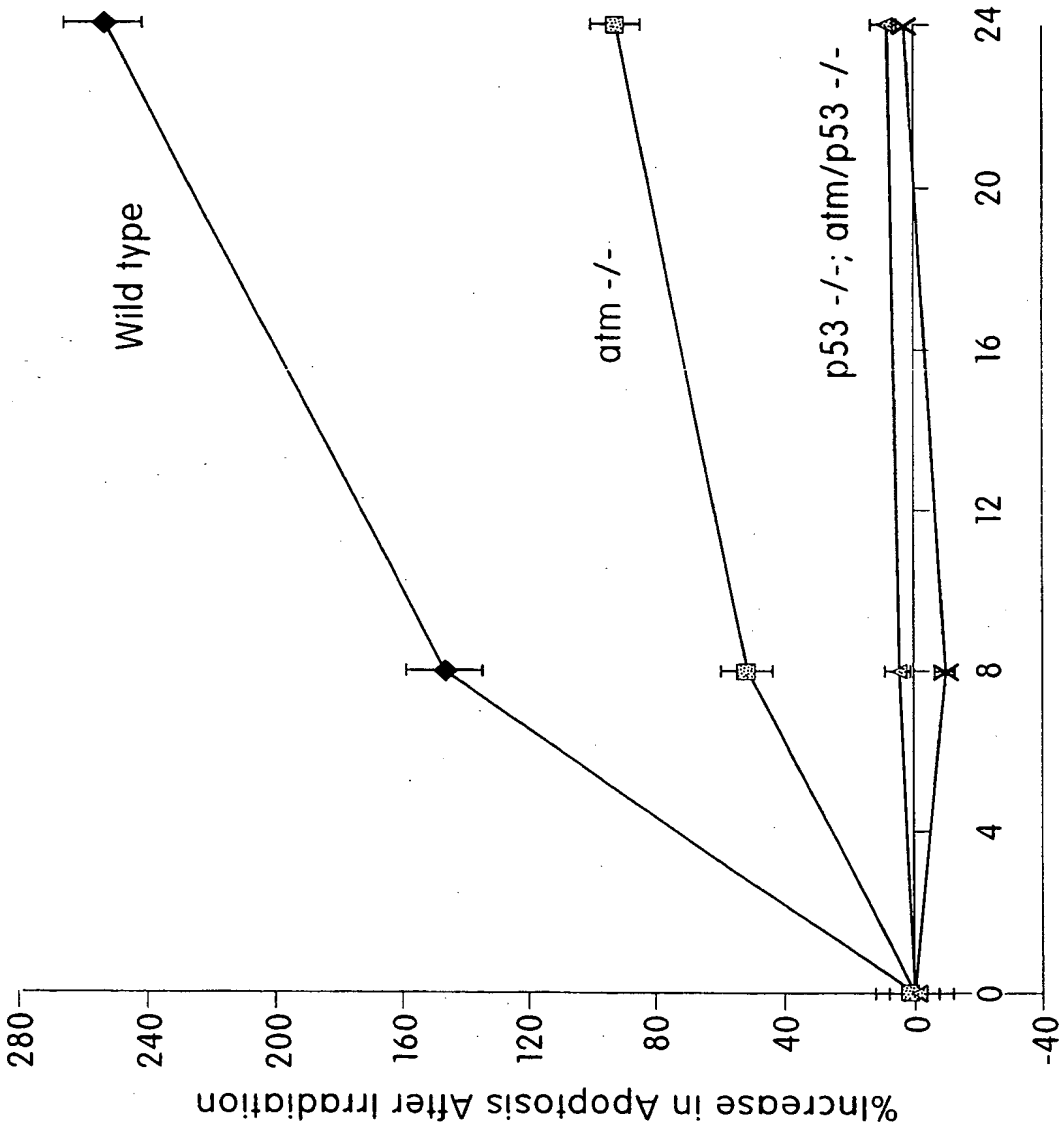


Fig. 1A

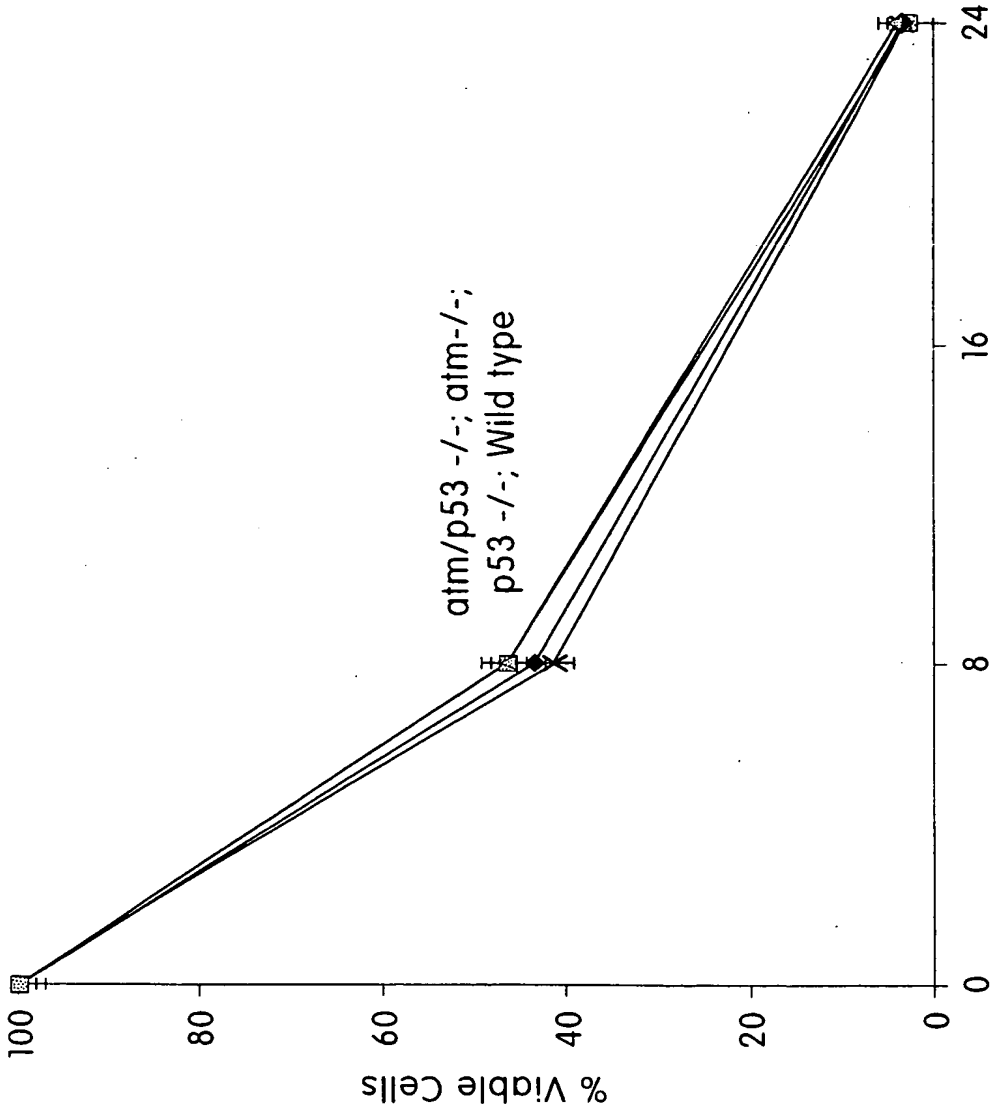


Fig. 1B

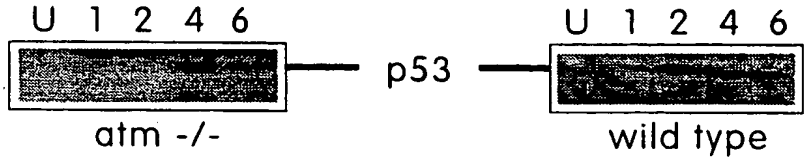


Fig. 2A

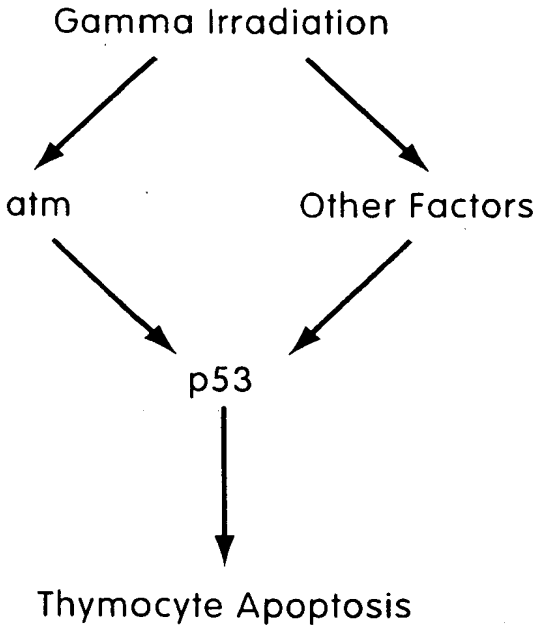


Fig. 2B

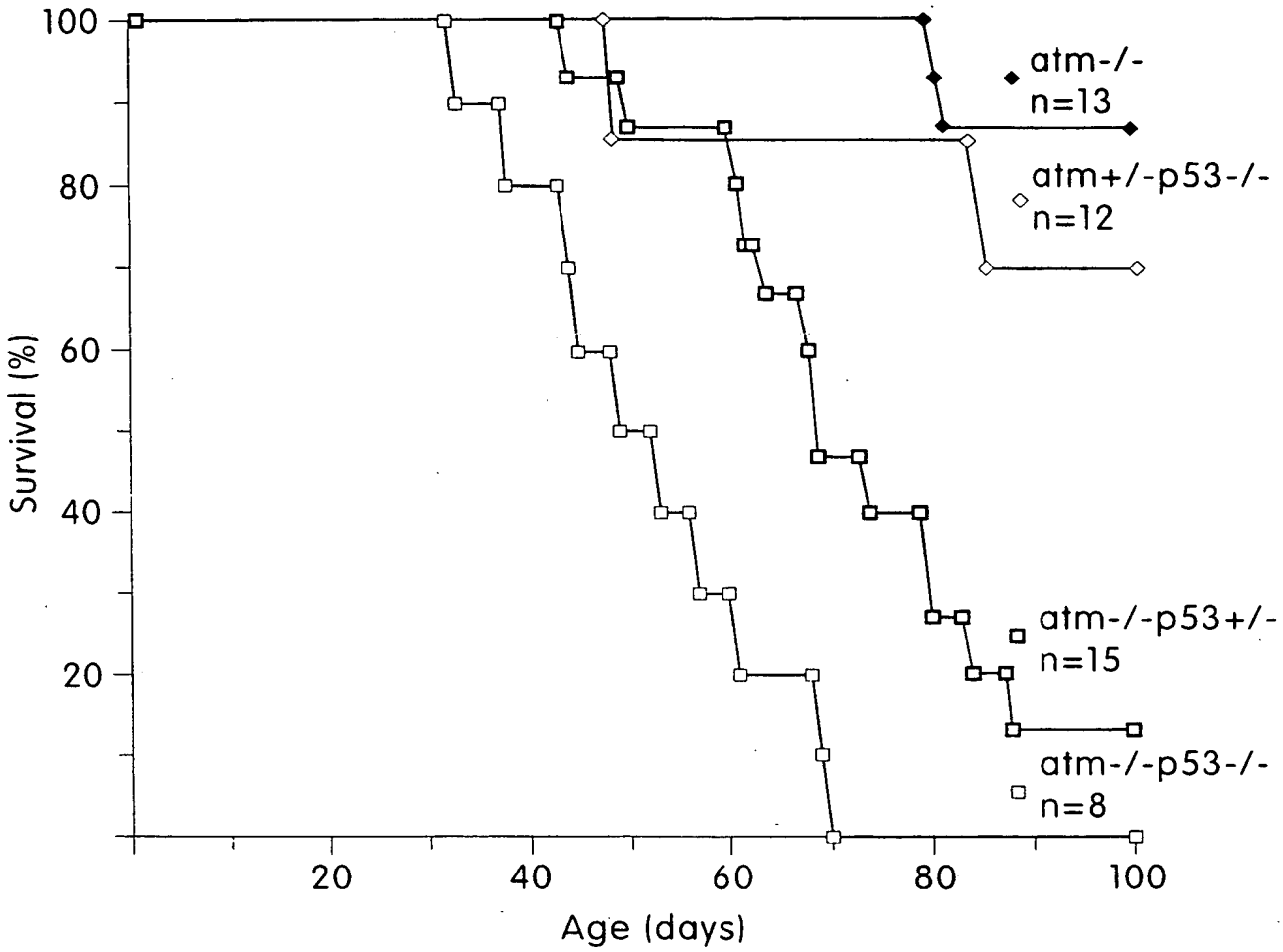


Fig. 3A

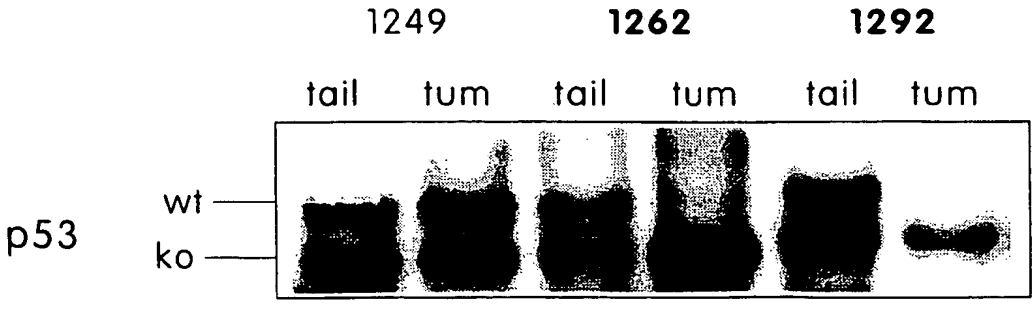


Fig. 3B

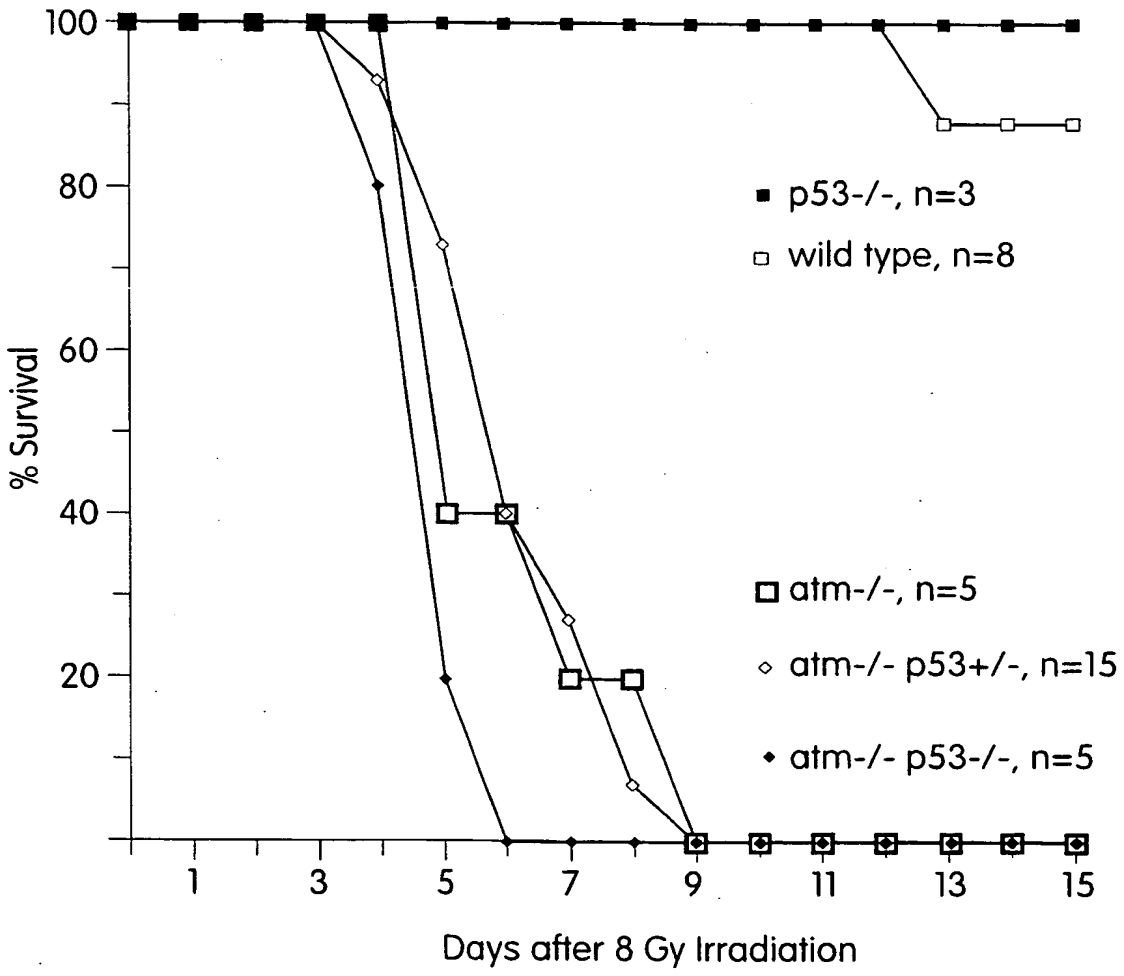


Fig. 4A

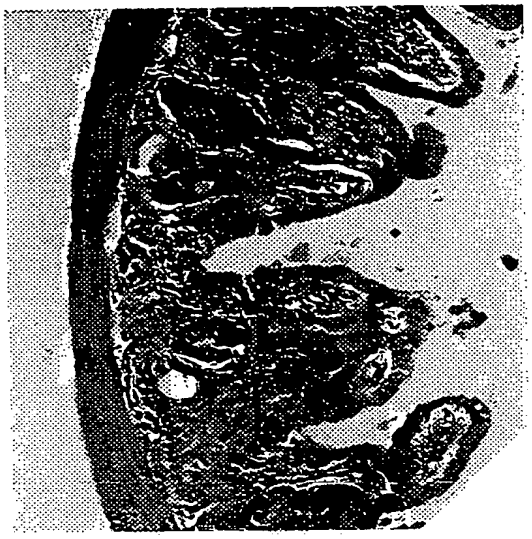


Fig. 4D

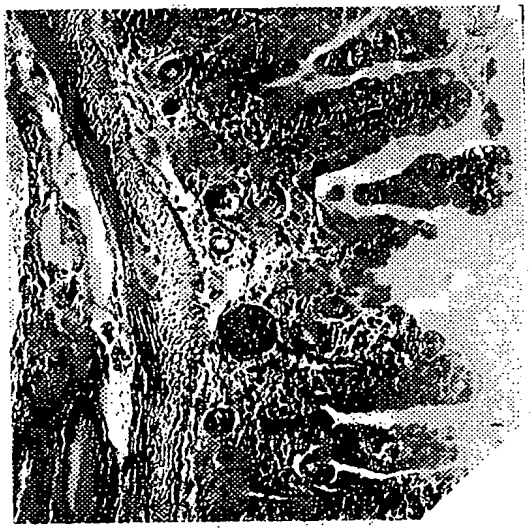


Fig. 4C

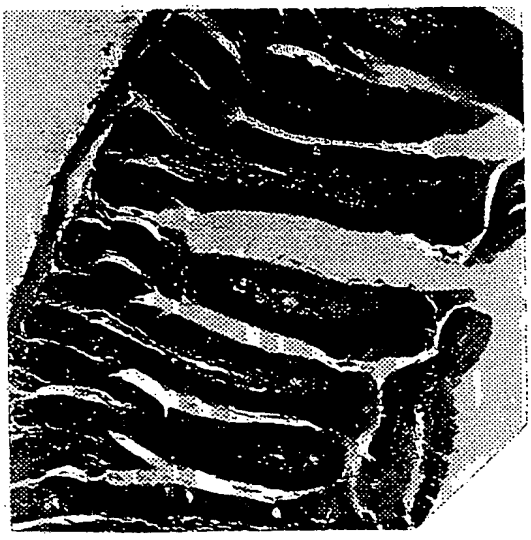


Fig. 4B

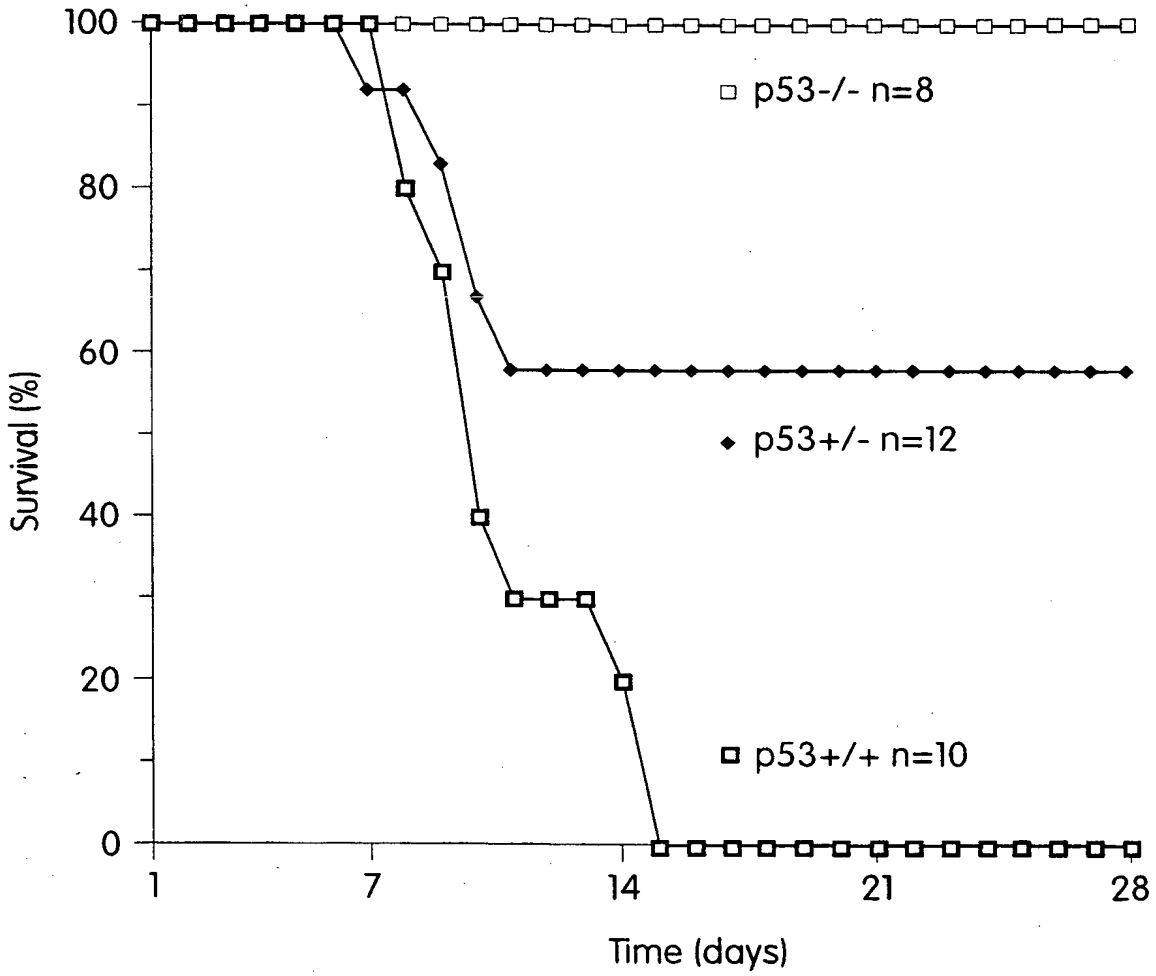


Fig. 5

Fig. 6B

Fig. 6D

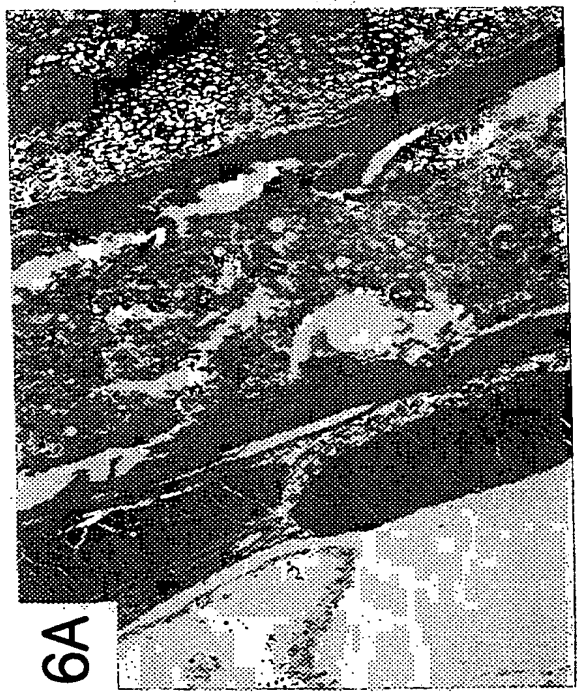


Fig. 6A

Fig. 6C

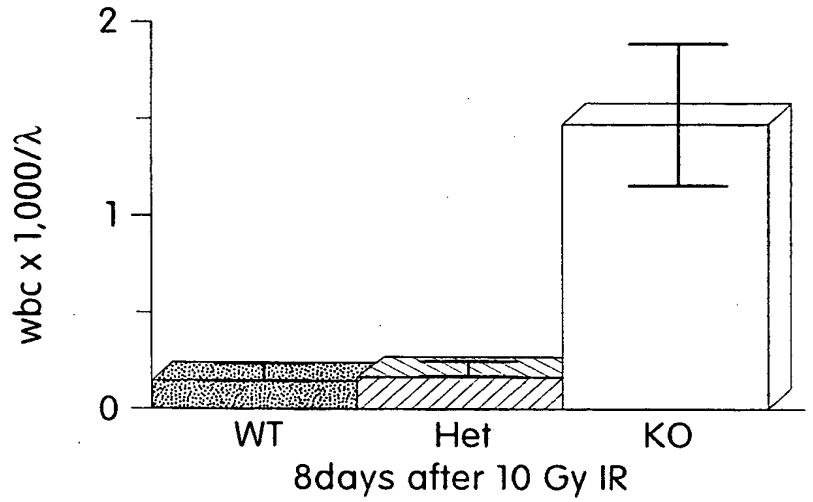


Fig. 7A

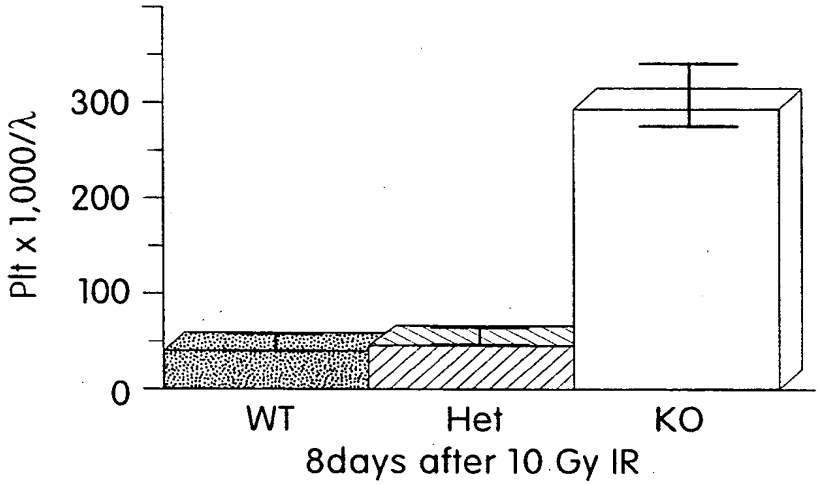
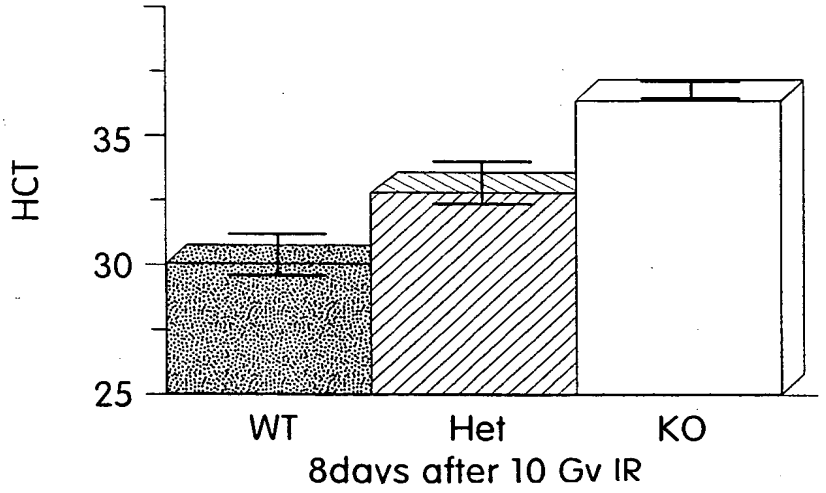


Fig. 7B



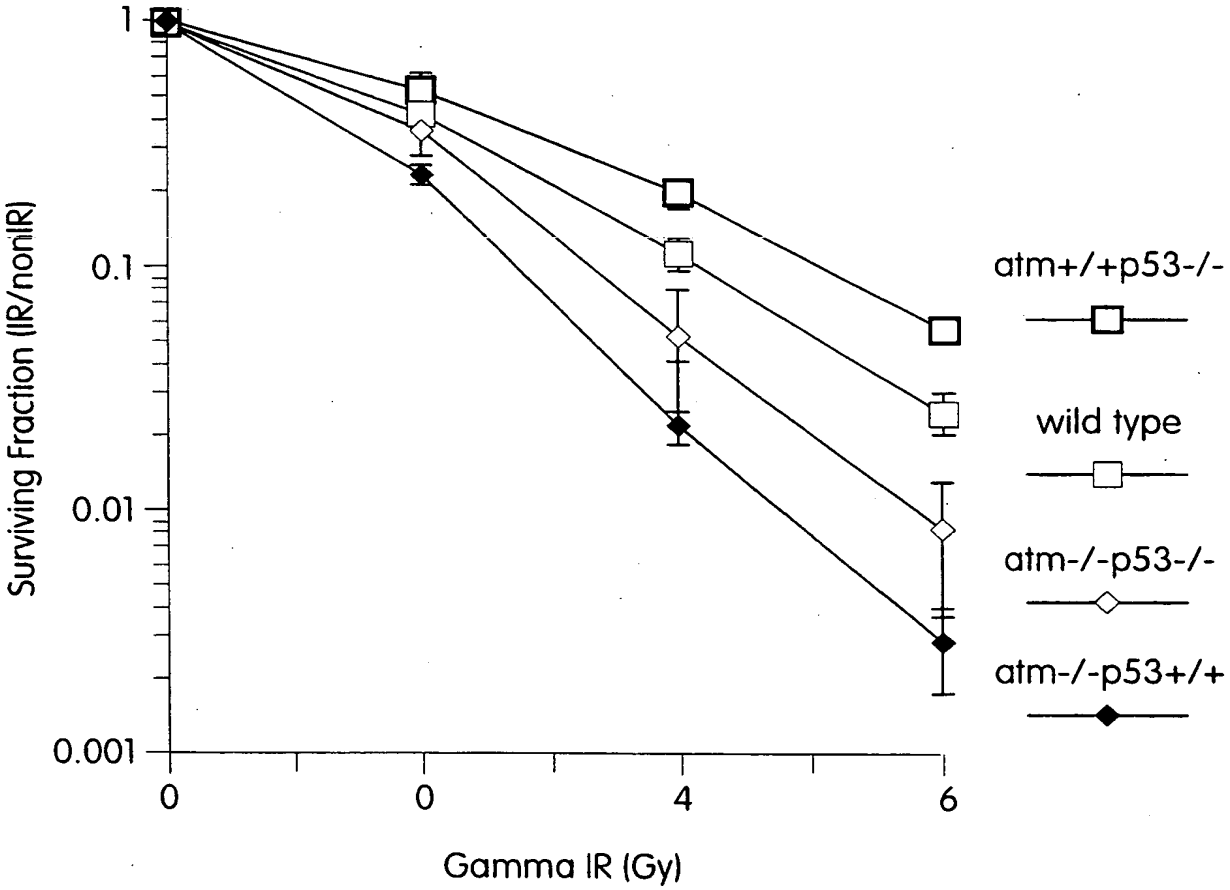


Fig. 8

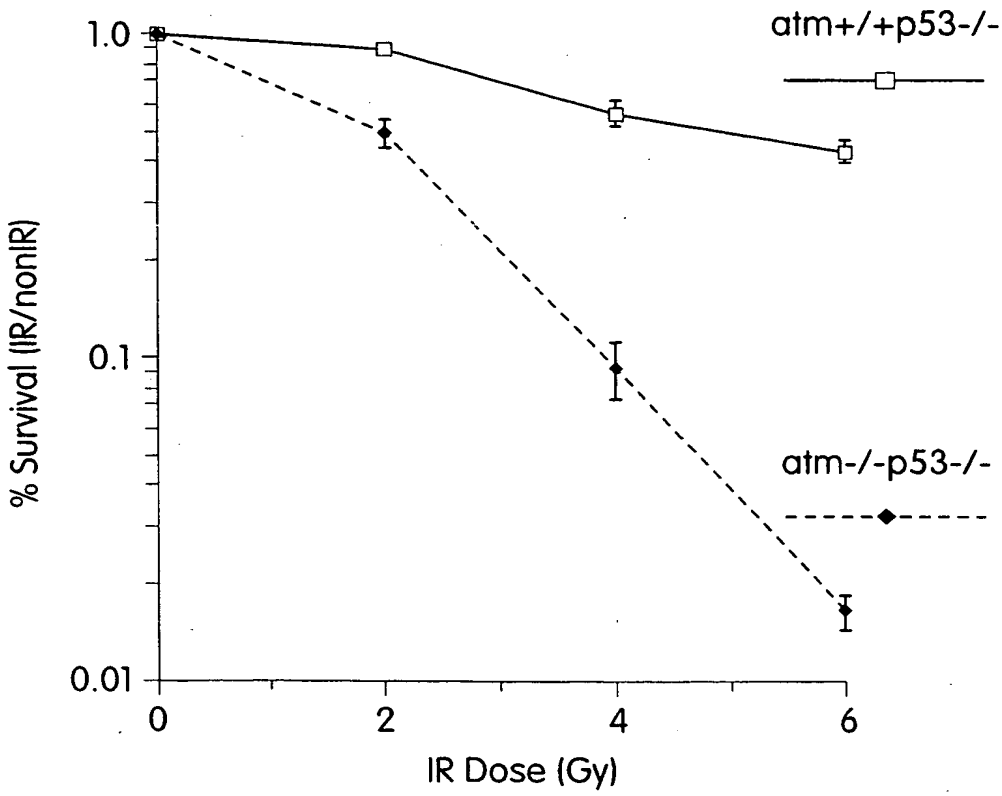


Fig. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12217

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 31/70, 39/395; A01N 43/04; G01N 33/574
US CL : 424/138.1, 155.1; 514/44; 435/7.23

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/138.1, 155.1; 514/44; 435/7.23

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ENOCH, T. et al. Cellular Responses to DNA Damage: Cell-cycle Checkpoints, Apoptosis and the Roles of p53 and ATM. Trends in Biochemical Science. October 1995, Vol. 20, No. 10, pages 426-430, especially page 427	1-12
A,P	XIE. G. et al. Requirements for p53 and the ATM Gene Product in the Regulation of G1/S and S Phase Checkpoints. Oncogene. 1998, Vol. 16, pages 721-736, especially page 721.	1-12
Y	JONGMANS, W. et al. The Role of Ataxia Telangiectasia and the DNA-dependent Protein Kinase in the p53-mediated Cellular Response to Ionising Radiation. Oncogene. 1996, Vol. 13, pages 1133-1138, especially page 1133.	1-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 AUGUST 1998	Date of mailing of the international search report 11 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer YVONNE EYLER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12217

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	XU, Y. et al. Dual Roles of ATM in the Cellular Response to Radiation and in Cell Growth Control. Genes & Development. 1996, Vol. 10, pages 2401-2410, especially page 2401.	1-12
Y	HAWLEY, R. S. et al. Strange Bedfellows in Even Stranger Places: the Role of ATM in Meiotic Cells, Lymphocytes, Tumors, and its Functional Links to p53. Genes & Development. 1996, Vol. 10, pages 2383-2388, especially page 2383.	1-12